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Correspondence From:

Last Name	First Name
Zon	Leonard

Remarks:

Please coordinate NIH response. Assigned to NCRR, NICHD, & NIGMS to provide input for response.

Subject:

Seeks funding for zebrafish research.

Your Program Analyst is **Barbara Williams**

Children's Hospital Boston
Director, Stem Cell Program
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May 1, 2006

Dr. Elias A. Zerhouni
NIH
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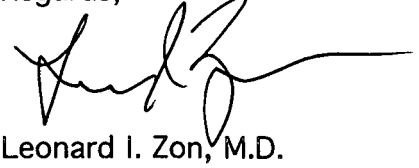
Building 1 - Shannon Bldg, Roo 126
1 Center Dr.
Bethesda, MD 20814

Dear Elias,

Hope all is well.

I wanted to contact you as a Representative of the zebrafish community. Recently, at a meeting in Maine, the primary investigators of the field met to develop a large-scale plan to expand the zebrafish as a model for human disease. Our proposal aims to develop novel strategies for understanding the pathogenesis of human disease and for drug discovery. I've included this "white paper" that was vetted by the zebrafish community (attached). In this plan, we propose to generate 2000 independent mutant lines in orthologs of human disease genes, and use transgenic technology to model other human diseases. With these models in hand, chemical screening will proceed to find pharmaceuticals that modify the disease. Compared to other animal models, the zebrafish system has the major advantage of not only modeling disease but looking for genes or drugs that suppress the disease itself. This large-scale project will become an important part of the RoadMap, and we believe deserves support from the NIH. I propose to meet with you to discuss this plan, and I would be happy to bring several senior members of the field. I believe that this plan will transform the study of disease.

Regards,



Leonard I. Zon, M.D.

The Zebrafish & Disease Project: Zebrafish as a model system to study and cure human diseases

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During the past 20 years zebrafish has served as an excellent model for understanding normal development and birth defects based on its powerful genetics and exquisite embryology. More recently, research with zebrafish has extended to model human diseases and to analyze the formation and functions of cell populations within organs. This work has generated new human disease models and has begun to identify potential therapeutics, including genes that modify disease states and chemicals that rescue organs from disease. For example, recent breakthroughs made in zebrafish include the isolation of a human skin color gene, the development of a melanoma model, and the isolation of a chemical that can correct cardio-vascular defects. Building on the exceptional intellectual and collegial openness of the zebrafish research community, a tremendous number of new investigators with disease-specific interests have been welcomed and rapidly trained in the field.

A recent meeting of the zebrafish community's primary investigators (September 14-17, 2005) led to a plan to create a discovery engine that will change the way we understand human diseases and treat them. This large-scale project will create mutations in zebrafish orthologs of human disease genes, generate transgenic animals for phenotypic analysis and disease modeling, and use chemical genetics to find therapeutic agents. Importantly, this project is not feasible or cost-effective in any other vertebrate model system. This plan will create a platform to study and cure human diseases and also benefit other research areas, from stem cells to neural circuitry. Below is a plan describing the community's objectives.

1. Reverse genetics: Generate mutations in zebrafish orthologs of human disease genes.

A number of strategies in the zebrafish system rely on forward genetics. This entails making a mutation and then finding the gene. Over the past three years, techniques to derive mutant zebrafish using reverse genetic strategies have also proven beneficial. For instance, the process of targeted lesion detection (or TILLING) has uncovered mutant models of human diseases. This strategy has identified p53 mutant zebrafish that have a predisposition to cancer and rag1 deficient zebrafish with immunodeficiency. Creating mutant zebrafish lines by TILLING, retroviruses or transposable elements dramatically simplifies disease modeling because of the powerful cellular and genetic approaches available in zebrafish to monitor disease progression. Moreover, suppressor and enhancer screens on these disease models, both using forward genetics and chemicals, provide excellent ways of modifying the disease phenotype. Such studies can identify genes that interact to produce models of multigenic human diseases and to identify drugs to treat them. A major advantage of the planned work in zebrafish will be the ability to generate a large range of gene changes that reflect the spectrum of alleles that predispose to disease in humans or only inactivate a gene product at a particular temperature. The zebrafish research community seeks funding to create a knock-out and allelic series for 2000 genes linked to human disease and key signaling pathways. Mutant zebrafish lines will be generated by a consortium of centers and distributed by a stock center. Data will be accessible on the zebrafish internet based information network, ZFIN (http://zfin.org), and NCBI. This project will provide a unique resource for both current zebrafish researchers and scientists in other fields.

2. Transgenesis: Generate tools for inducing and modeling human diseases in vivo.

The zebrafish offers the unique opportunity to follow normal and aberrant developmental and

physiological processes in vivo at subcellular resolution. In particular, transgenic animals can be continuously monitored and confocal imaging can be used to study behaviors of cell populations highlighted by fluorescent proteins in vivo and in real time. Recent improvements in transgenesis methods have made the zebrafish a dramatically more efficient and cost-effective system than the mouse. In addition, transgenic zebrafish have been used to create human disease models. This is particularly important, because many human diseases are caused by the ectopic expression of genes. For example, a transgenic zebrafish carrying the activated BRAF gene implicated in human skin tumors leads to large melanomas. The zebrafish research community proposes to develop several thousand zebrafish strains that mark every tissue and cell type of the body and that allow the misexpression of human disease genes. This can be achieved by generating transgenic lines in which a reporter or disease gene is expressed under the control of a tissue- or cell type-specific promoter. To generate a system that allows the tissue-specific expression of any protein of choice, several thousand transgenic lines will be generated that express an inducible transcription factor or recombinase in specific tissues and cells and that can activate the expression of any gene of interest. These transgenic zebrafish lines will be generated by a consortium of laboratories and distributed by a stock center. Data will be accessible via ZFIN (http://zfin.org) and NCBI. This transgenesis project will provide in vivo markers for mutant screens and the study of disease progression at cellular and subcellular resolution, and generate models for human diseases caused by gene misexpression.

3. Chemical genetics: Find drugs that suppress diseases.

The recent development of the zebrafish as a model for chemical genetics has established chemical screening in vivo as an adjunct to older screening technologies in cell lines or in vitro. Soluble chemicals permeate into zebrafish embryos and produce specific effects. For instance, 50 – 70% of the chemicals known to cause abnormalities of the cell cycle in mammalian cell lines also affect the zebrafish cell cycle *in vivo*, and drugs blocking prostaglandin synthesis are as effective in zebrafish as in humans. In contrast to screening by *in vitro* techniques, zebrafish offers an *in vivo* vertebrate model for studying the bioactivity of chemicals. In addition, the availability of large numbers of zebrafish mutants makes chemical suppressor screens fast and straightforward. For example, one screen identified a chemical that suppressed a specific cardiovascular disease. The zebrafish research community proposes to assign two centers to (1) distribute chemical libraries to individual labs in a format for zebrafish experiments and (2) perform large-scale screens for chemicals that suppress genetically caused diseases. The centers will provide fish holding and screening/imaging facilities for zebrafish researchers and could undertake more than 24 large scale screens with up to 100,000 compounds and over 200 individual screens with about 2000 bioactive compounds. Data will be collected by ZFIN (http://zfin.org) and NCBI. The targets of chemicals found to prevent or cure disease phenotypes in zebrafish will, in general, have very close cognates in humans. Therefore these screens promise to provide key entry points for the development of new therapeutic drugs.

Summary

In summary, the zebrafish provides a unique advantage for large-scale vertebrate genetics combined with exceptional visualization of cell populations and physiological processes. The ability to make models of human diseases coupled with suppressor and enhancer screens and chemical screens will make the zebrafish an even more important contributor to our understanding of human disease. The proposed projects will complement the ongoing NIH initiative that supports inventive screens and tool development (PAR-05-080). The zebrafish primary investigators agree that the combination of reverse genetics, forward genetics and chemical genetics will provide fundamental insights into human diseases and their cure.

National Institute of Neurological Disorders and Stroke (NINDS)
William Talbot (Stanford University)

The zebrafish is now a premier model system for the study of development and function of the vertebrate nervous system. Unique experimental advantages of the zebrafish system include the optical clarity and accessibility of the embryo, the availability of large collections of mutations disrupting the essential genes, and the relative simplicity and rapid development of the nervous system. Despite its simplicity, the zebrafish nervous system shares many fundamental similarities with other vertebrates, including the patterning of the neural tube, the control of neural and glial differentiation, the positions of axon tracts, saltatory conduction by myelinated axons, regeneration and degeneration, and many aspects of behavior. Thus insights from studies in zebrafish usually apply directly to higher vertebrates, including humans.

In the past five year, a host of exciting findings have emerged from the combination of the powerful cellular and genetic approaches available in zebrafish. Some examples of recent discoveries in zebrafish include: new components of the Notch and Hedgehog signaling pathways; understanding the cellular interactions that coordinate left-right asymmetry in the brain; miRNAs are essential for brain morphogenesis; synapses stabilize dendritic processes and axonal branches; activities of competing neurons regulate dendritic field size in the trigeminal ganglion and the in the tectum; chemokines regulate sensory ganglion assembly; Schwann cells regulate sensory organ differentiation; ErbB receptors direct Schwann cell migration; Heparan sulfate proteoglycans are essential for sorting of retinal axons in the optic tract; and application of cAMP can induce regeneration of severed axons in the CNS.

Previous Trans-NIH Initiatives to generate genomic resources and promote focused mutant screens enabled many of the successes listed above, and these programs have also set the stage for exciting advances over the next few years. For example, a number focused screens in the zebrafish community have identified mutations in genes essential for: development of certain populations of neural stem cells, myelin and the nodes of Ranvier, synapse formation and the regulation of synaptic activity, neuronal migration, and behavior. These mutations will provide key insights into the formation and function of the vertebrate nervous system. Moreover, phenotypic and molecular studies indicate that these mutants will serve as models of debilitating human conditions, including neurodegenerative disease, stroke, hypoxia, multiple sclerosis, peripheral neuropathies, and spinal cord injury.

Improvements in transgenic technologies and imaging methods are rapidly advancing the analysis of the zebrafish nervous system. For example, several groups have generated collections of GFP enhancer traps expressed in specific patterns in the nervous system. Moreover, fluorescent reporters of neural activity allow functioning neural circuits to be monitored in normally behaving zebrafish. The combination of transgenic reporters and the exquisite imaging possible in the zebrafish promises to provide a unique window into the nervous system over the next few years: the subcellular locations of fluorescent proteins and organelles will be traced in neurons and

glia during development, behavior, and regeneration. This will revolutionize our understanding of many dynamic processes in the nervous system, including axon outgrowth, synapse formation, partitioning of axons into the nodes of Ranvier and other domains, trafficking of cargo and signaling molecules along axons, and the repair of damaged axons. The combination of these new transgenic markers and disease models will provide a wealth of knowledge about the pathophysiology of diseases of the nervous system in human.

High-throughput chemical screens in zebrafish will provide a key step toward the design of therapies for diseases of the nervous system. Zebrafish models are available for many human diseases and injuries involving the nervous system, and screens for new mutations will increase the number further. Water-soluble compounds can readily be applied to zebrafish, and their activities may be rapidly monitored by a number of approaches, including behavior and fluorescent transgenic reporters that distinguish wild-type from disease states. By screening for small molecules that suppress the mutant phenotype, each zebrafish mutant can become a platform for drug discovery. The combination of zebrafish mutants, fluorescent reporters, and small molecule screens holds great promise for the development of therapeutics that can prevent and repair damage to the nervous system.

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National Institute of Mental Health (NIMH)
Herwig Baier (UCSF)

A growing number of laboratories are using zebrafish for the study of neuro-psychiatric disorders, often with NIMH support. This is not surprising, given the unique experimental advantages of this model system for neurobiological research:

The zebrafish brain is similar to the mammalian brain. The basic organization of the CNS is highly conserved among all vertebrates, including humans. Several groups have mapped out the distribution of neurotransmitters and neuromodulators in the zebrafish CNS in great detail. The growing list of well-substantiated similarities between fish and humans now includes peptidergic systems, such as parathyroid 2, opioids, and FMRF, as well as aminergic systems, such as dopamine, serotonin, and histamine [1-9]. Thus, insights gained from the study of synaptic circuitry and transmitter physiology in zebrafish is broadly applicable to humans.

Zebrafish may be used for the discovery of behavioral genes. Zebrafish are amenable to large-scale forward-genetic screens focusing on behavior. Unlike screens in the mouse, zebrafish screens can be carried out by individual R01-funded labs. Robust behavioral assays have been developed, including conditioned place preference and forms of non-associative learning. Several behaviors can be scored in high throughput mode, allowing comprehensive screens for mutations or for bioactive chemical compounds. Hundreds of behavioral mutants have so far been discovered. Their mutant phenotypes include defects in habituation, sensitization, prepulse inhibition, motor regulation, visually guided behavior, circadian rhythms, and sleep [10-23] Many of these phenotypes have direct relevance to human neuropsychiatric diseases, such as addiction, schizophrenia, and sleep disorders.

Reverse genetics can model neuropsychiatric disease mechanisms. Zebrafish can also be used for "reverse genetics" to test the function of candidate genes by morpholino knockdown or TILLING. This possibility is now being exploited for verification of genetic modifiers in heterogeneous complex disorders, such as Gaucher's disease (E. Sidransky, NIMH). Importantly, zebrafish mutants of a known disease-susceptibility locus could also be used as a sensitized genetic background for the *unbiased* identification of modifier genes. This approach constitutes a powerful combination of reverse and forward genetics, not possible in any other vertebrate.

The zebrafish CNS is optically accessible. Young zebrafish are small and optically transparent, enabling functional imaging studies in an intact brain at unrivalled resolution. Transgenic zebrafish lines may express reporters of circuit development and function. GFP and its variants, including genetically encoded indicators of synaptic activity, such as cameleon, are increasingly being used to label neuronal cell types and to monitor their activity in the brain of living animals [24-32].

Zebrafish develop rapidly. The zebrafish brain develops within less than a week after fertilization to a functional organ, which then fully supports the survival of a swimming, food-seeking animal. Psychiatric conditions, such as schizophrenia, autism, and Tourette's

syndrome, have been associated with neurodevelopmental disorders. Hypotheses concerning the consequences of developmental perturbations on behavior can be tested quickly in zebrafish. For example, NIMH grants are allowing H. Sive's group (MIT/Whitehead) to study the genetic control of brain ventricle inflation, as well as the consequences of failed inflation on brain function [33].

Small size and rapid development of zebrafish allow high throughput compound screens. Screening assays can frequently be performed in 96-well plates (with one or a few fish in each well). This feature allows automatic liquid handling and phenotype scoring, and consequently high throughput[34]. With funding from the NIMH, the University of Pittsburgh – Molecular Libraries Screening Center has therefore added zebrafish to its preferred model systems for high throughput compound screens (G. Lazo, PI). For instance, a zebrafish reporter line is now used to search for drugs that promote formation of oligodendrocytes [35].

Electrophysiology in the zebrafish CNS is rapidly catching up. Recently, the labs of M.-m. Poo (UC Berkeley), F. Engert (Harvard), and H. W. Tao (USC) have succeeded in patch-clamp recordings of neurons in the intact zebrafish CNS (unpublished work). This technical breakthrough is now allowing electrophysiology to be carried out on zebrafish mutants with neurodevelopmental or behavioral defects and has thus removed one of the perceived weaknesses of the zebrafish preparation for neurobiological research.

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National Institute of General Medical Sciences (NIGMS)
Alexander Schier (Harvard University)

Research funded by the NIGMS aims to provide fundamental insights into life processes and to develop new tools and techniques. The zebrafish is an ideal model system to contribute to this mission, because it is highly accessible for embryological and genetic analysis.

Signaling. Zebrafish research has made important contributions to our understanding of signaling pathways during vertebrate embryogenesis. In particular, the genetic dissection of Nodal, FGF, BMP, Hedgehog and Notch signaling has led to the discovery of new components in these pathways: the EGF-CFC protein One-eyed pinhead is a coreceptor for Nodal and Lefty signals; the Sizzled protein Ogon is negative regulator of BMP signaling; the interleukin-17-like protein Sef antagonizes FGF signaling; the extracellular protein You enhances hedgehog signaling; the ubiquitin ligase mind bomb is required for Delta/Notch signaling. The embryological and molecular analysis of zebrafish mutants has provided a detailed framework for how signals and transcription factors interact to regulate vertebrate embryogenesis. This includes the discovery of a genetic cascade that drives endoderm formation, the realization that Nodal/Lefty signaling constitutes a reaction-diffusion system in embryonic patterning and the finding of novel roles for retinoic acid signaling during left-right and heart development.

Morphogenesis. Zebrafish research has also contributed to the understanding of morphogenesis and cell migration. For example, genetic and cellular analysis of non-canonical Wnt signaling has provided genetic evidence that this pathway is required for convergence and extension and neurulation and has revealed novel roles for this pathway in linking cell division and morphogenesis; Cxcr4 has been identified as a G-protein coupled receptor that guides germ cells.

MicroRNAs. Most recently, studies in zebrafish have made important contributions to microRNA biology. The analysis of zebrafish microRNAs has revealed exquisite expression patterns and led to the discovery of a role for miR-430 in clearing maternal mRNAs by inducing their deadenylation.

Technology development. Zebrafish research has also pioneered technological developments, including the establishment and use of large-scale genetic screens to identify mutations affecting zebrafish development and physiology; the use of morpholino antisensense oligonucleotides as reagents to block mRNA splicing or translation in vivo; the design and use of retroviruses and transposable elements for large-scale gene disruption in vertebrates; the application of TILLING, a method to create an allelic series of mutations in any given gene; the use of confocal imaging of fluorescent markers for high-resolution in vivo analysis of gene function; and the screening of small molecules to identify novel modulators of cellular processes and potential therapeutic drugs.

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National Institute of Environmental Health Sciences (NIEHS)
Robert L. Tanguay (Oregon State University)

It is firmly established that human health is significantly impacted by interactions with environmental factors. Broadly defined, environmental factors include the air we breath, the food we eat, the water we drink, the pharmaceuticals we ingest, and the daily stress we endure. Importantly, individual susceptibility to environmental insult is modified by endogenous factors such as genetic variability and by age. The mission of the National Institute of Environmental Health Sciences (NIEHS) is to understand the important interactions between environmental factors and individual susceptibility, and with this increased knowledge to reduce adverse health outcomes. Multidisciplinary and integrative approaches are essential to fill these information gaps. With the current tools in hand, there is a tremendous opportunity to exploit the unique advantages of zebrafish to improve human health.

Molecular and Developmental Toxicology – It is largely accepted that vertebrates are more responsive to environmental insult at the earliest life stages. The probable molecular explanation for increased embryonic susceptibility is that there is no other period of an animal's life span when the full repertoire of molecular signaling is necessary and active. The implication of this hypothesis is that if a chemical is developmentally toxic, it must interfere with, or modulate, a pathway conserved between disparate species and at different life stages. So, a practical side of studying environmental responses during development is to protect the most susceptible human populations, but just as importantly, this life stage has the greatest potential to identify signaling modulators. Thus, the best system for elucidating underlying mechanisms of environmentally-induced toxicity or disease would be during early development. As proof of principle, NIEHS has supported research aimed at establishing zebrafish as a model to understand the interactions between 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and the developing vertebrate. Using classic toxicology, biochemistry, genetic and molecular approaches, researchers have clearly demonstrated that the physiological and molecular responses to TCDD are conserved across taxa. With this understanding, there are numerous opportunities to use zebrafish to improve human health in a number of additional areas. Specific opportunities are briefly described below.

Rapid throughput small molecule efficacy and toxicity screening. During the post genomic era there will be an ever increasing emphasis on the development of novel drugs for the treatment of human diseases. There is a pressing need to develop rapid, relevant, cost effective *in vivo* models to asses the efficacy and toxicity of small molecules. Zebrafish have proven to be useful as part of the overall strategy in drug discovery. Similarly, the assessment of environmental contaminants as potential toxicants is routine, and the enormously complex interactions between chemicals in mixtures can now be effectively evaluated using zebrafish.

Identification and validation of biomarkers of exposure. There are significant advantages in using comparative toxicogenomic approaches in zebrafish to identify biomarkers of exposure or biomarkers of disease. First exposures can be more precisely controlled in respect to dose and developmental stage, but more importantly, once identified, the role of identified biomarkers can be rapidly assessed using molecular and genetic techniques. Approaches such as gene repression and over expression are routine, rapid, and effective in zebrafish.

Chemical genetics. Diverse natural and synthetic chemical libraries are available and are being screened for bioactivity in zebrafish. Efficient *in vivo* screening will allow the identification of cellular pathways perturbed or modified by chemical exposure, and offer opportunities in target identification. In addition, suppression of disease by novel chemicals has the potential to clarify underlying mechanism. These approaches have the real potential to efficiently lead to diagnostic or therapeutic interventions.

Transgenic reporter lines. Tissue specific, or conditionally regulated reporter transgenic lines are readily available to identify the biological target of environmental chemicals. For instance, transgenic reporter expressing fluorescent proteins have proven useful in assessing target cell toxicity in live animals. There are numerous potential applications of transgenic reporter animals including gene expression or proteomic analysis of cell sorted populations, genetic screens for modifiers of molecular responses using reporter genes as convenient endpoints, *in vivo* evaluation of protein-protein interactions etc, to name a few.

Epigenetics. There is mounting evidence to suggest that environmental chemicals have the potential to epigenetically modify genes, and that these genetic modifications may contribute to disease etiology. Using the unique advantages of the zebrafish model, complex epigenetic mechanism may be elucidated *in vivo.*

In vivo evaluation of gene function. In the post genomic era, evaluation of the role of gene products in the biological responses to environmental insult will be an important, but immense challenge. The role individual or groups of genes in disease processes can be efficiently and cost effectively evaluated in zebrafish as part of an integrative applied research approach.

Environmental influences on CNS development and function. An emerging area of concern is the impact of low level human exposures to environmental contaminants on CNS development and function. The possibility that early life stage exposures may lead to persistent effects on learning, memory, or behavior has not been adequately addressed using other vertebrate models. This is largely due to the experimental limitations of rodent models. There is enormous opportunity to use zebrafish to fill this large information gap.

Identify modifiers of environmental response. Genome wide genetic screens are now routine in zebrafish, however there is a significant untapped potential to use forward or reverse genetics to identify genetic modifiers of susceptibility to environmental insult. Since chemical mutagenesis is non-biased, there is a high probability that currently unknown targets will be identified. The identification of novel genes can be rapidly evaluated using integrated approaches in mammals.

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National Institute on Deafness and Other Communication Disorders (NIDCD)
Teresa Nicolson (Oregon Health and Science University, Portland)

Neurobiologists are increasingly turning to zebrafish to answer basic questions in neuroscience, from the level of peripheral sensory receptors to the development and function of neural circuits. The possibility of unbiased forward genetic screens combined with newly developed techniques such as gene-knockdown, TILLING, and transgenic expression offer the ability to examine the molecular basis of peripheral and central nervous system function in intact animals. In addition to identifying molecules and gaining insight into their functions in neurons, elucidating the etiology of human diseases such as human congenital deafness is facilitated by studying the zebrafish model.

As seen in recent studies, orthologues of human deafness genes are being identified in forward genetic screens for auditory and vestibular mutants in larval zebrafish. Among the zebrafish genes cloned to date are myosin VIIa, myosin VI, cadherin 23, and protocadherin 15 (Ernest et al., 2000, Seiler et al., 2004, Kappler et al., 2004, Söllner et al., 2004, Seiler et al., 2005). Mutations in these genes cause congenital deafness in humans and there are some corresponding mice models (shaker-1, walzer, and Ames waltzer). The phenotypes in mice and zebrafish are very similar, demonstrating conservation of function. So why bother with zebrafish mutants? In one study already using zebrafish, the relative ease of screening for mutants has yielded an important result. The isolation of hypomorphic alleles of cadherin 23 (cdh23) in zebrafish has identified a functional role for Cdh23 in mechanotransduction in hair cells, a conclusion not possible with the currently available mouse models. In the gating-spring hypothesis of hair-cell mechanotransduction, extracellular filaments known as tip links are thought to pull open transduction channels when hair bundles are deflected in the excitatory direction. The molecular nature of the fine filaments at the tips of hair bundles was unknown since their discovery by anatomists two decades ago. Work conducted using zebrafish provided evidence that Cdh23 is a protein constituent of the tip link (Söllner et al., 2004). The discovery of Cdh23 as the tip link will require rethinking the current gating-spring model of transduction. Many cartoons draw the tip link as an elastic spring. Cadherins, however, are not known to be elastic, suggesting that the spring element may be located elsewhere. A reverse-genetic study in zebrafish (Sidi et al., 2003) has also revealed a candidate for the hair-cell transduction channel, TRPN1, first discovered in Drosophila sensory bristle mutants (Walker et al., 2000). Interestingly, mice and humans do not have this gene, however, a recent study proposed that a similar channel, TRPA1, is the transduction channel in mouse hair cells (Corey et al., 2004).

In addition to large-scale forward genetics, imaging and experimentation with live cells in intact animals is only possible in zebrafish. In mammalian or chick models, it is necessary to remove and dissect the tiny ears of embryonic or newborn animals, a laborious and tedious process. The advantages of the transparency of the inner ear and accessibility of superficial hair cells (lateral line organ found in fish and amphibians) cannot be overstated. Studying inner ear development in zebrafish has led to our current understanding of the molecular basis of such basic processes as otic induction (Whitfield et al., 2002). The generation of transgenic lines expressing specific markers of the

auditory/vestibular system or mutated forms of proteins will yield further insights into the development and function of the ear and neural circuitry involved in hearing and balance. As these tools are continually created and exploited, the popularity of the zebrafish as a model for human health and disease will undoubtedly continue to grow.

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National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)
Iain Drummond (MGH)

Along with the NICHD, the NIDDK co-chairs the NIH-wide Zebrafish Coordinating Committee which has overseen zebrafish genomics projects and the development of tools to further advance the fish as a genetic model. In addition, the NIDDK currently supports research consistent with the goals of these Institute program areas:

Basic Renal Biology and Developmental Biology of the Kidney and Urogenital Tract
The zebrafish pronephros is now established as a relevant model of kidney development and disease. Studies of the early development of the pronephros (Drummond, DK53093) have defined its structure and morphogenesis, highlighting equivalences between fish and mammalian kidney cell types and regulatory factors. In vivo functional assays of novel transcription factors that pattern the nephron and are essential for the differentiation of tubules and podocytes (Schultheiss/Drummond, DK071041; Davidson, MGH, Boston; Hukriede, University of Pittsburg) take advantage of the simple bilateral and linear pronephric nephrons. Cystic mutants (Sun, DK069528; Drummond, DK53093) have identified multiple novel genes involved in cystic pathology and defined a role for cilia in maintaining nephron fluid flow. Genes known to play a role in human cystic disease (MODY5, PKD2, NPHP2, BBS) are also being directly studied in zebrafish and Medaka (Sun, DK057328; Drummond, DK070263; Obara, DK069604; Burdine, Princeton University; Fisher, Johns Hopkins). Nephropathy, proteinuria and the function of podocyte slit-diaphragm proteins have also been modeled in the fish (Drummond, DK53093). This assay system has been successfully used to implicate a novel gene (mosaic eyes/B4.1L5) in the process of podocyte junction formation. The fish is also being employed to rapidly screen the function of many novel podocyte-specific transcripts for a potential role in mammalian podocyte pathology (Majumdar, Karolinska Institute, Sweden). The zebrafish kidney has also been established as a model of acute nephrotoxic injury (Hentschel, Brigham and Women's Hosptial, Boston) and shows promise as a quantitative model of renal clearance that could be adapted to high-throughput screening. The potential for the zebrafish kidney to regenerate new nephrons after nephrotoxic injury is remarkable and highlights the value of this model for studies of tissue stem cells and cell-based therapies (Davidson, MGH, Boston).

Bone and Mineral metabolism. Mutants affecting adult skeleton and bone structure have been isolated and analyzed radiographically (Fisher, Johns Hopkins). Defective skeletogenesis and kidney stone formation observed in the zebrafish touchtone/nutria mutants have recently been shown to be caused by mutations in the trp channel trpM7 (Parichy, UT Austin). The feasibility of using fish for high throughput screening of bone anabolic compounds has also been recently demonstrated (DanioLabs, Cambridge UK).

Cancer. The unique advantage of being able to screen thousands of small molecule compounds in living whole zebrafish larvae has been exploited to identify suppressors of zebrafish cell cycle mutants (Stern, DK061849). This approach holds great promise for developing therapeutics that perturb specific oncogenic pathways.

Endocrinology. The genetics of pituitary corticotroph development are being actively pursued in a mutagenesis screen employing a novel zebrafish reporter transgenic line that drives

expression of GFP from the pro-opiomelanocortin gene promoter (Liu, DK064806). These studies will shed new light on the poorly defined genetic pathways involved in pituitary cell differentiation.

Gut, Liver and Biliary, Pancreas. Differentiation of the endoderm and its derivatives is a very active area of zebrafish research. The regionalization and specification of endoderm derivatives by retinoic acid and other secreted factors offer the potential of directing stem cell differentiation into specific endoderm cell types (Prince, DK064973). Genetic screens for novel endoderm mutants (Stainier, DK058181) and studies of how the pancreatic primordium is specified by Hox proteins (Sagerstrom, DK068237) will further characterize molecular pathways involved in endodermal organogenesis. Studies are also underway to specifically examine genes that may direct pancreatic beta-cell differentiation (German/Stainier, DK061245) and regulate the level of insulin synthesis (Stoffers, DK068157). Lineage and specification of the exocrine pancreas and intestine is also currently being analysed genetically by mutagenesis screening (Pack, DK061142; Mayer, DK002968) and in studies of the musashi RNA binding protein and the PTF1a-p48 transcriptoin factor (Leach, DK056211, DK067210). Liver development has been analysed in a classical genetic screen employing a novel GFP endoderm reporter transgenic line (Stainier, DK060322); the genes isolated in this screen are now being actively positionally cloned. A study of the specific role of wnt signaling in liver neoplasia (Goessling, DK071940) is taking advantage of the ability to engineer inducible expression of wnt pathway effectors in zebrafish and the availability of other mutant lines with defective APC and p53 tumor suppressor genes. The pescadillo gene, originally identified in zebrafish insertional mutagenesis screening, is being analysed for its role in liver growth and regeneration (Duncan, DK060064). GI tract physiology and the genetic determinants of GI motility are being pursued in zebrafish (Rich DK071588) and offer the potential to better understand human gut motility disorders. Specific defects in the enteric nervous system have also been revealed in zebrafish mutants (Shepherd, DK067285). The transparency of the zebrafish larva and the use of novel quenched fluorescent lipid is facilitating the analysis of biliary development and transcription factors that drive this process (Matthews, DK068009). Specific fluorigenic substrates of PLA2 have been employed in mutagenesis screening to identify novel regulators of gut PLA2 (Farber, DK060369) an important enzyme of the intestinal brush border. Interactions of indigenous gut microbiota and gut epithelial cells are being studied (Guillemin, DK067065) to test whether the zebrafish can be used to model opportunistic infections, inflammatory bowel disease and atopic allergies in humans.

Hematology. The cloning of many mutations in zebrafish hematopoiesis by the Zon lab (Orkin/Zon, DK049216) has lead to many novel insights into erythropoiesis and myelopoiesis. This ongoing work and work from other labs is exploiting GFP reporter lines and hematopoietic mutant lines to study novel genes co-regulated with known hematopoietic transcription factors GATA-1 and GATA-2 and genes that are absent in mutants (Lin, DK054508). Cell interactions that drive blood cell fate decisions are being pursued in the spadetail mutant where erythropoiesis, but not myelopoiesis, is defective (Ho, DK068286). Conversely, four genes have been identified that specifically affect myeloid development by screening insertional mutants for myeloperoxidase activity (Rhodes, DK069672) and these are being further characterized. Novel genes regulating iron uptake have been identified in mutagenesis screens (ferroportin1, Donovan, DK064924) and new loci affecting iron transport are being positionally cloned (Fraenkel, DK061685). Characterization of zebrafish as a model of adult hematopoietic stem cell

isolation and differentiation is being pursued (Traver, DK066254) exploiting the unique ability to perform cell transplantation and fate mapping in living zebrafish embryos.

Genetics and Genomics of Kidney Urologic and Hematologic Diseases. The NIDDK has been a leading institute in developing the genetic and genomic infrastructure for zebrafish research. Currently supported initiatives include continuation of RH mapping (BAC contigs) and coordination of the genome assembly (Zon, DK055381), core repositories for hematopoietic mutants (Zon, DK049216), development of gene trap vectors and gene targeting systems (Lin, DK065638), generation of GFP gene trap transgenic lines (Johnson, DK069466), genetic mapping resources for zebrafish (Smith, DK065637), and finally, automation systems for zebrafish fluorescent lipid assays (Ferrante, DK068887).

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National Institute on Drug Abuse (NIDA)
John Dowling (Harvard University)

Little is yet known about the genetics of drug addiction and abuse. Having a vertebrate animal with which one can readily do forward genetics and that responds positively to addicting drugs would be exceptionally useful. Although little has been done so far with zebrafish and addicting drugs, the results to date suggest zebrafish can serve as model organism for such research.

It has been shown, for example, that zebrafish can be readily place-conditioned to cocaine or other dopamine releasing drugs such as the amphetamines. That is, if zebrafish are exposed to a source of cocaine that is positioned at one end of an elongated tank on one day, they will preferentially return to that end of the tank on subsequent days when there is no cocaine present. This is the classic conditioned place-preference (CPP) response. Using this response as an assay for sensitivity to cocaine, Darland and Dowling (2001) isolated three dominant mutants that have an altered sensitivity to cocaine. A more recent study has shown that genetic impairment of acetylcholinesterase dramatically reduces the place-condition response of zebrafish to D-amphetamine (Ninkovic *et al.*, 2006)

At the present time, NIDA is supporting just two grants involving zebrafish and addicting drugs. Both studies involve cocaine, so it is unknown whether zebrafish will respond to other types of addicting drugs, but such studies clearly seem warranted. One of the two studies with cocaine is focusing mainly on the genetics of cocaine responsiveness, the other on the effects exposure to cocaine have on wild-type fish. The latter study, for example, is examining whether zebrafish develop behavioral sensitization to cocaine, whether they exhibit withdrawal effects and so forth.

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National Institute of Child Health and Human Development (NICHD)
Marnie Halpern (Carnegie Institution Baltimore)

From the outset, the National Institute of Child Health and Human Development (NICHD) has been a strong supporter of advancing the zebrafish model and promoting new initiatives for genomic tools and gene discovery. The zebrafish was quickly recognized as a powerful system for genetic and cellular studies of embryonic development and growth. This research has led to insights into human disease, birth defects and potential therapies. For example, based on the work in zebrafish, EGF-CFC proteins have been found to be associated with human birth defects, the analysis of zebrafish neurulation defects suggests novel causes of neural tube defects in humans, and the dissection of embryonic signaling pathways guides attempts for the in vitro generation of organs or specific cell types and informs stem-cell based therapies.

Zebrafish has also proven to be a valuable model for structural birth defects, notably craniofacial disorders and syndromes involving multiple organ systems. For example, improper specification of the dorsal-ventral axis, through disruption of early signals or their propagation, can lead to an excess of blood cells, loss of specific neuronal cell types and reduced muscle. NICHD has supported basic studies on how the body axes are set up and the identification of the molecular pathways involved. Comparative approaches are especially instructive, in that establishment of the left-right axis, which underlies morphological asymmetry of the heart and visceral organs, has aspects that are conserved and that differ between vertebrate species. Antisense methodology allows rapid testing of candidate regulatory cues in zebrafish. Additionally, the zebrafish system has provided some of the first molecular tools for probing differences between the left and right sides of the vertebrate brain.

Developmental studies have also focused on the differentiation of specific cell types and the mechanisms whereby their numbers are tightly regulated. This has led to new insights into how mesodermal derivatives interact to promote red blood cell formation and how the neural crest generates tissue diversity and correct cartilage morphology. Once specified cells must recognize and adhere to appropriate neighbors for tissue integrity, and ultimately functional specialization. In the zebrafish hindbrain, where this is being rigorously described, cells at distinct anteroposterior levels respond to a different array of signals and maintain segmental identity through unexpected cell-cell interactions. Cell recognition also is an essential component of synaptic connectivity; zebrafish mutational strategies continue to reveal new players in axon guidance and pathfinding.

Cellular specification, differentiation, recognition and morphogenesis are only part of the story of how organs achieve their correct shape and size. The zebrafish fin serves as a useful model for exploring the regulation of growth. A number of new mutations affect the mode of fin elongation during development, resulting in reduced growth or overgrowth. Identification of affected genes is revising how tissue interactions are thought to control growth.

NICHD funded projects have often focused on research problems that are more challenging or impossible to pursue in other model systems. For instance, using forward genetic screens, new initiatives are aimed at characterizing ovary development and oogenesis, or the intriguing process of metamorphosis.

An important future direction is to increase our understanding of how single gene disruptions as well as multilocus effects can produce complex developmental syndromes. Zebrafish models for human disorders, such as DiGeorge and Bardet-Biedl Syndrome, are allowing new insights into tissue specificity and genetic interactions that may underlie the pleiotropic and variable phenotypes that are observed clinically.

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National Institute of Biomedical Imaging and Bioengineering (NIBIB)
Alexander Schier (Harvard University)

The transparency of zebrafish embryos and larvae make this model system ideally suited for biomedical imaging studies. Developmental and physiological processes can be observed at the single-cell and even subcellular level in a live animal. The power of the system has been exploited to provide important insights into vertebrate biology.

Recent advances include the visualization of gastrulation and neurulation movements. These studies revealed that differential adhesion, directed cell division and cell intercalation contribute to embryonic morphogenesis. In vivo imaging of germ cell migration uncovered that these cells are guided towards their targets by the chemokine SDF-1a and its receptor Cxcr4/Odysseus.

Imaging studies in the nervous system revealed mechanisms underlying neuronal migration and differentiation, axon pathfinding and growth, and synapse formation. Studies in organ systems have allowed the in vivo imaging of physiological processes. For example, zebrafish larval kidneys have motile cilia that drive fluid flow, as shown by videomicroscopy of injected fluorescent tracers. An assay for the uptake of fluorescent lipids by the gut identified genes that regulate lipid metabolism. The analysis of fluid flow during cardiac development has revealed an important role for fluid forces in shaping the heart.

Other recent advances include the analysis of protein in trafficking in embryonic tissues, the use of genetically engineered calcium indicator cameleon to record neuronal activity *in vivo*, and the development of selective plane illumination microscopy, which allows optical sectioning through thick live samples with minimal photobleaching.

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National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS)
Monte Westerfield (University of Oregon)

Recent research has demonstrated that zebrafish muscle, bone, and skin development and function are strikingly homologous to that of humans, at tissue, cell, and molecular genetic levels. Mutant analyses have uncovered dozens of genes required for muscle development, genomic sequencing has identified zebrafish orthologs of musculoskeletal disease genes and where tested these orthologs play homologous roles. For example, blocking genes underlying muscular dystrophy cause similar symptoms in zebrafish (Bassett et al., 2004; Parsons et al., 2002; Nixon et al., 2005). Zebrafish models of other human diseases including HyperCKemia, rippling muscle disease and distal myopathy have also been developed (Nixon, et al., 2005). In zebrafish the powerful genetic and cellular approaches not available in any other vertebrate can offer powerful tools to deepen our understanding of these and other musculoskeletal and skin diseases.

Research with zebrafish has also resulted in completely new discoveries about the mechanisms underlying normal development of muscle. Mutations that affect the specification of skeletal muscle subtypes, slow or fast, have revealed that members of the Hedgehog family of signaling proteins regulate this key step in development (Kawakami et al., 2005; Wolff et al., 2003). Because of the power of zebrafish genetics and embryology we have learned with single cell resolution how muscle lineages form and lead to the population of adult muscle (Hirsinger et al., 2004). These studies pave the way toward development of diagnoses and therapies for human myopathies. The excellent progress in our understanding of muscle and connective tissue development and disease will continue to depend on advances in zebrafish genomics.

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National Institute of Allergy and Infectious Diseases (NIAID)
Len Zon (Harvard Medical School)

The zebrafish has been an excellent system to study the immune system. Zebrafish, being a vertebrate, have all the lineages of hematopoietic cells that mammals have. In addition, the system allows a genetic manipulation and transgenic visibility unrivaled in the current models of the immune system. Genetic screens have been done to look for immunodeficient zebrafish and comparative approaches from a variety of aspects of the immune system have been proven valuable. The zebrafish develops its lymphocytes at day 4 of life and have fully developed immunoglobulin and T cell receptor production. The ontogeny of the thymus has been well studied by morphology and electronmicroscopy. Gene expression studies with a variety of molecular probes delineate the onset of thymogenesis at 4 days of development. Transgenic zebrafish have been created which target lymphocyte populations with green florescent proteins. This includes a rag2 promoter driving GFP, which is expressed in B cells as well as T cells, and the LCK promoter driving GFP, which is involved in T cell development. The LCK promoter and rag promoter have been used to drive oncogenic fusion proteins and the animal develop lymphoma. The marrow of the zebrafish is in its adult kidney. Flow cytometry analysis of forward scatter and side scatter delineates the lymphoid population within the marrow. This has been used to analyze a variety of different immunodeficient mutants.

Genetic screens have been performed to find mutants that lack T cells. This includes a screen in which rag 1 was used as a probe for T cell development. Several genes have been isolated that are defective in T cell development. One of the genes, *van gogh*, proves to be mutated in the TBX1 ortholog. This TBX1 has been implicated as the gene involved in DiGeorge Syndrome. A comparative approach between DiGeorge patient samples, immunodeficient mice and TBX1 deficient zebrafish mutants is being used to delineate conserved pathways involved in craniofacial development and immune system oncogeny.

The zebrafish is amenable to small molecule screening. A proof of principle has been done in which lymphocytes are lysed in the presence of dexamethasone. This can be visualized using the GFP lines, and high throughput chemical screens can be done to look for new immunomodulatories. The interplay between the host response and pathogens is likely to be an area of focus with the zebrafish. The first studies examined fluorescent tuberculosis and the ability of macrophages to engulf the tuberculosis in vivo. Other studies have been done with salmonella and e coli in similar processes. Pathways involved in neutrophil and monocyte migration patterns can be visualized in vivo. In addition, mutants can be used to evaluate organism application. For instance, a mutant that lacks myeloid cells does not replicate tuberculosis, whereas a mutant that lacks T cells does. This helps better understand the host response.

The TOLL receptors are present in the zebrafish and a number of investigators have begun to probe NFkB signaling in the zebrafish. This leads to an evaluation of the

zebrafish as a model for other immune diseases. Several genes including AIRE and FOXD3 are being targeted for this approach.

Hematopoietic stem cells biology continues to be a focus in the zebrafish field, with marrow transplants being successful, and the evaluation of graft vs. host effects and leukemia is underway. Adaptive immunity that creates autoimmunity has been studied in the zebrafish. In summary, the zebrafish is an excellent system for the study of the immune system. Being a vertebrate at the evolutionary beginning of the onset of the immune system proves to have some advantages in terms of understanding basic immune function. In addition, the application of disease mutants to chemical biology should provide new therapeutic opportunities.

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National Institute on Alcohol Abuse and Alcoholism (NIAAA)
Michael Carvan (University of Wisconsin-Milwaukee)

The societal costs related to alcohol abuse are in excess of $200 billion annually for lost wages, crime, property damage and social services (Harwood, 2000). Nearly 1% of all live births are affected by fetal alcohol spectrum disorders, including fetal alcohol syndrome, as a result of developmental alcohol exposure. The cost to society is estimated to exceed $5 billion annually for health care, developmental disabilities services, special education and lost wages for affected individuals (Riley and McGee, 2005). Susceptibility to alcoholism and fetal alcohol spectrum disorders has a significant genetic component and the mechanisms by which alcohol exerts its effects are poorly understood.

Zebrafish have great potential to serve as a model for understanding the mechanisms by which alcohol effects behavior and for revealing the biochemical pathways that influence addiction. Adult zebrafish exposed to alcohol show dose-dependent changes in locomotor activity, aggression and socialization that mimic behavioral changes in humans. Moderate alcohol increases movement, aggression and sociability; whereas higher exposures lead to decreases in all these behaviors. Alcohol addiction in zebrafish has not yet been reported; however, the behavioral paradigms being developed by several laboratories are easily adapted to such analyses and the molecular toolbox available should support strong mechanistic inquiry.

The effects of developmental alcohol exposure in zebrafish have been under investigation for a number of years and recent results demonstrate that zebrafish responses are remarkably similar to those of mammalian models and humans. Zebrafish efficiently metabolize alcohol and excrete its metabolites using the same enzyme systems as other vertebrates, and correlation between exposure concentration and tissue dose has been established. At high doses of alcohol, skeletal morphogenesis of the facial and cranial structures is impacted, often leading to cyclopia, and abnormalities in a number of other structures, including the axial skeleton, heart and limbs, are readily apparent. The expression of *shh, axl,* and *nk2.2* in the ventral aspects of the forebrain and midbrain is also significantly altered. These developmental defects are more subtle at lower alcohol doses but are still significant within the range found in the blood of adult alcoholics. Exposure of zebrafish embryos to lower levels of alcohol also causes neurobehavioral abnormalities in young animals and learning disabilities in adults even when treatment is limited to the first 24 hours post-fertilization. Strain dependent differences in alcohol susceptibility have been demonstrated and suggest that zebrafish is an excellent model to identify genes that influence susceptibility and resistance to the effects of developmental alcohol exposure.

The zebrafish has proven to be a valuable model for genetic analysis of the molecular and biochemical pathways that influence vertebrate development in addition to several adult phenotypes. Recently, a number of phenotypes in zebrafish have been defined that relate to alcohol abuse and the effects of developmental alcohol exposure (teratogenesis and neurobehavioral dysfunction). Investigators are beginning to undertake mechanistic studies of alcohol-induced behavioral impairments, organ damage and teratogenesis. Further development of the zebrafish model should include studies focused on the identification of genes that influence alcohol-related phenotypes using mutagenesis screens, microarray

analysis, and mapping of loci associated with susceptibility or resistance. Functional analyses of these genes will significantly contribute to our understanding of alcohol-related disease processes in humans.

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National Institute on Aging (NIA)
Monte Westerfield (University of Oregon)

Zebrafish are well known as a powerful model for genetic studies in developmental biology. Recently, the zebrafish system also has given insights into several human disorders including cancer and neurodegenerative, hematopoietic, and cardiovascular diseases. Because aging processes are related to these and various other human disorders, several groups have started studies of senescence in zebrafish as a model for human aging.

To characterize aging in zebrafish and to provide a baseline for comparisons with humans, several aging biomarkers are used. Beta-galactosidase activity in skin and oxidized protein accumulation in muscle are associated with senescence in both humans and zebrafish (Kishi, 2004). These biological and biochemical aging markers already characterized in normal zebrafish are currently being used in analyses of transgenic lines and in genetic mutant fish screens. Recent analysis has shown that Tis21(Btg2/Pc3), the endogenous cell death molecule and pan cell cycle regulator, provides a link between cellular senescence and carcinogenesis (Lim, 2006). Analysis of the heat shock response in aging zebrafish has also revealed new insights into p53 function and how cells change their responses to stress with aging (Keller, 2004). These and similar efforts will help to elucidate the functions and molecular mechanisms of common pathways of aging among vertebrates from fish to humans. Other work is directed toward identifying specific targets in disease-associated pathways, such as osteoporosis and Alzheimer's Disease. Identification of the genetic pathways that regulate senescence and zebrafish models of senescence will significantly contribute to the discovery of potential drugs applicable to age-associated diseases.

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National Heart, Lung, and Blood Institute (NHLBI)
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The developing cardiovascular system is extremely accessible in zebrafish allowing unprecedented access to imaging and manipulation. For these reasons, the zebrafish system has become increasingly popular in the cardiovascular field. Significant advances have been made using this model system in issues of cell differentiation, morphogenesis and organ function.

Formation and function of the heart
Heart formation The promise of stem cell biology to treat various forms of heart disease relies partly on our ability to differentiate stem cells into cardiomyocytes. Advances in zebrafish and other model systems are bringing us closer to such manipulations. However, much information remains to be uncovered including the identity of the extracellular signals during this differentiation process. The zebrafish model is at the forefront of these studies.
The complexity of cardiac morphogenesis is reflected in the prevalence of congenital heart malformations, and studies in zebrafish have provided new insights, genes and signaling pathways into this process. For example, a recent study clearly demonstrated the influence of cardiac function on cardiac morphogenesis. In addition, the relative simplicity of the zebrafish cardiovascular system compared to its mammalian counterpart will allow the investigations of cardiac form and function to proceed to the single cell level (and probably at subcellular resolution), a level of resolution needed for a satisfying comprehension of the biology of these processes.

Heart Regeneration Zebrafish hearts, unlike mammalian hearts, regenerate. The long-term vision of this work is that a cellular and molecular understanding of the regenerative process in zebrafish will help design therapies to help the diseased mammalian heart heal and possibly 'rejuvenate'.

Heart Function Many common cardiac disorders are difficult to model in mammals. Despite strong evidence for heritability, the responsible genes are largely unknown, and difficult to discover in humans. For example, dilated heart failure is a common disorder, frequently "idiopathic" in nature, with strong familial predisposition. Several zebrafish mutants exhibit the same dilated, poorly contractile physiology as do these patients. Other mutants model common rhythm disorders, including heart block, fibrillation, or sinus bradycardia. In addition, the accessibility of the zebrafish heart allows the screening of drugs for adverse effects on cardiac function.

Blood vessel biology
Blood vessel development is key to numerous biological processes including organ growth and repair, and an imbalance in angiogenesis contributes to numerous malignant, inflammatory and ischemic disorders. The zebrafish system continues to contribute significantly to our understanding of how endothelial cells develop, assemble into tubes and navigate through their environment. In addition, high throughput methods have been used to identify chemical suppressors of genetic defects in blood vessel

formation. On the basis of these studies, it is safe to predict that the zebrafish system will also contribute significantly to our understanding of endothelial cell homeostasis, a necessary step in the design of better drugs to treat or prevent adult-onset vascular diseases.

Sleep

Sleep is an emerging area of interest in the zebrafish community. And clearly again, the ability to use forward genetics to investigate such a basic biological question is bound to uncover new insights.

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National Human Genome Research Institute (NHGRI)
Shawn Burgess (National Human Genome Research Institute)

It can be argued quite effectively that the value of a model organism to biomedical research is deeply enhanced by the quality and breadth of its genomic resources. This is doubly true in cases (such as zebrafish) where genomics can be coupled with genetics. There has been a strong and coordinated effort within the community of zebrafish researchers to provide comprehensive genomics resources. These efforts are directly in line with many of the essential elements of NHGRI's mission statement.

The lead on sequencing the zebrafish genome was taken by the European scientific community and the majority of this effort has taken place at the Wellcome Trust Sanger Institute in Cambridge (1). There are (and were) several US efforts in support of this genome project. The BacPac consortium has generated several zebrafish BAC libraries as both an essential resource for sequencing the genome and for researchers utilizing BAC recombination to create transgenic zebrafish (2). This includes a "doubled-haploid" BAC and fosmid libraries that were made to solve genome assembly issues that arose from the very high polymorphism rates of zebrafish. There were radiation hybrid panels coupled with gene and EST mapping efforts that laid the essential framework for genome assembly (3). Intimately linked to the genome sequencing efforts is the Zebrafish Gene Collection or ZGC (4). This is a full-length cDNA sequencing project hosted by the NIH and containing 7,053 sequences to date. This information is critical to zebrafish researchers to rapidly take advantage of new discoveries. In parallel with the ZGC is the NIH funded effort to define the early spatio-temporal expression of all zebrafish genes by high-throughput *in situ* hybridization (5). This effort will open up new avenues for researchers to data-mine quickly to identify important candidate genes. Finally, a great deal of the genetic and genomic information is collected and curated by the Zebrafish Information Network (6), a centralized zebrafish information website funded by NHGRI. This site allows researchers to seamlessly move through information on genes, mutations, gene expression, and publications among other things. This has created a comprehensive set of genomics tools that profoundly impacts all other branches of zebrafish research.

With the continually improving genomics resources, approaches that fundamentally rely on extensive genomic information become possible. Targeted knockouts known as reverse genetics, is a technique that requires good understanding of gene structure in order for the techniques to be effective. Morpholino inhibition is commonly used in zebrafish for targeted knockdown and genomic information allows groups to approach knockdowns in a functional genomics approach. An NIGMS grant funds a screen to knockdown all putative secreted proteins in zebrafish (7). This is impossible without good genomics tools. Similarly TILLING or resequencing from mutagenized genomes is being used to identify mutations in specific genes. This is a technique that allows researchers to obtain stable mutations that can be tested in adult fish, which is not possible using morpholinos. Two large projects are funded by NHGRI to generate TILLING/resequencing libraries (8,9). Other approaches for zebrafish reverse genetics, some funded by the NIH, that are currently underway include high-throughput mapping

of retroviral integrations (10,11) and gene/enhancer trapping efforts using retroviruses and transposable elements (12,13). Many of these approaches are similar to those taken in the mouse model, but zebrafish has an advantage of scale in that an order of magnitude more zebrafish can be housed in similarly sized facilities.

The potential for new insights gained by comparative genomic studies has been highlighted by several recent reports. For example, a haplotype analysis of the zebrafish pigment gene golden led to the discovery of a human gene that contributes to skin color. The in vivo analysis of regulatory elements between zebrafish and human revealed conservation of regulatory function despite lack of overt sequence similarity.

In terms of the future, genomics and functional genomics resources could focus on systematically generating stable mutations for every zebrafish gene. New technologies and approaches will need to be developed to achieve this. In addition, using microarrays or other technologies, transcriptional profiling of all zebrafish tissues would provide both a resource for comparative genomics across model systems and a deep datasource for data-mining new gene targets. Efforts such as these would require an integrated effort from the community but would provide a broad, stable foundation of genomic tools that can be used by the entire research community to accelerate biomedical research.

1) http://www.sanger.ac.uk/Projects/D_rerio/; 2) http://bacpac.chori.org/libraries.php; 3) Grant #: 3R01DK055378 (genome mapping); 4) http://zgc.nci.nih.gov/Info/Summary; 5) Grant#: 2R01RR015402 (high-throughput *in situs*); 6) http://zfin.org/; 7) Grant #: 5R01GM063904 (morpholinos); 8) Grant #: 5R01HG002995 (TILLING)
9) Grant #: 1Z01HG000102 (TILLING); 10) Grant #: 5R44HG002871 (retroviral mapping); 11) Grant #: 1Z01HG000183 (retroviral mapping); 12) Grant #: 5R01GM069382 (Tol2 transposable element); 13) Grant #: 5R01DA014546 (Sleeping Beauty transposable element)

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National Eye Institute (NEI)
John E. Dowling (Harvard University)

For eye and vision research, zebrafish offer several unique features over other model systems. For example, zebrafish are highly visual animals that respond readily to a variety of visual stimuli. Thus, visual responses can be easily elicited enabling investigators to measure visual performance with a high degree of precision. Another important advantage of zebrafish for visual studies is that its retina contains abundant cones as well as rods, and the cones are arranged in a precise mosaic pattern across the retina. Zebrafish are tetrachromatic possessing cones that absorb maximally in the red (575 nm), green (480), blue (415) and ultraviolet (360 nm) regions of the spectrum. Further, each cone type is morphologically distinct, enabling their identification (Robinson *et al.,* 1993).

Eye and retinal development are rapid in zebrafish; within 24 hours postfertilization (pf) a well-formed eye is present and differentiation of the retina occurs between 1 and 3 days pf (Schmitt and Dowling, 1994, 1999; Raymond *et al.*, 1995). By 4-5 days pf, quantitative visual responses can be elicited from the animals. Mutations that affect eye development and visual function can be readily isolated using forward-genetic techniques, and mutations that cause human visual system diseases, such as Usher's syndrome have been found and are being studied. Electrophysiological recordings from both the eye and optic tectum can be readily made and the effects of drugs and other chemicals on visual function and development can be studied, often by simply placing them in the water surrounding the animal or eye.

Presently, about forty (40) grants employing zebrafish are being supported by the National Eye Institute and initially all of them employ mutant animals or they intend to in the future. The largest numbers of grants (14) are investigating retinal and photoreceptor development. Six (6) grants are concerned with specific eye disorders including glaucoma, ocular neovascularization, age-related retinal degenerations and Usher syndrome. Six (6) grants are investigating the optic tectum and retinotectal projections, including retinal axonal pathfinding, the development of retinotectal projections and the basic physiology of the tectal neurons and their synapses. Five (5) grants are using zebrafish to uncover basic retinal anatomy and physiology, and four (4) grants are concerned primarily with zebrafish visual response behaviors and screening for visual system behavioral mutants. Three (3) grants are focusing on lens development and the structure and function of lens crystallins. Finally, two (2) grants are concerned primarily with retinal injury and regeneration.

At the present time, the number of mutations that have been isolated and that affect primarily eye and retinal function in zebrafish is probably close to 250. Recessive mutations that affect the photoreceptors and retinal development are most commonly found (Malicki *et al.,* 1996), but dominant mutations that cause slow retinal degenerations similar to retinitis pigmentosa in humans have been described (Li and Dowling, 1997). A sampling of the sort of mutations that have been found by behavioral

testing or by observation of the developing eye, as well as their usefulness, is provided below.

A behavioral mutant isolated about a decade ago was shown to be completely blind behaviorally, yet its retina appears perfectly normal histologically at both the light and electron microscopic level (Brockerhoff *et al.,* 1995). Electroretinographic (ERG) recordings showed photoreceptor activity (an a-wave), but no second-order cell responses (b- or d-wave). Brockerhoff and her colleagues have recently found the mutation is in the gene for a subunit of the pyruvate dehydrogenase (PDH) complex. Why a mutation in the PDH complex blocks photoreceptor synaptic transmission is not yet clear, but these mutant animals are proving useful for the analysis and search for therapeutic cures for a human disease. PDH deficiency is found in humans, causes severe brain and eye defects, and leads to early death (Taylor *et al.*, 2004). Current treatments for humans have met only limited success and an animal model for this disease is of great value for testing therapeutic approaches. Brockerhoff and her colleagues have now rescued the mutant animals by adding ketogenic substances to the water in which the embryo develop and have shown both restored vision and survival of the animals.

Another behavioral mutant causes the selective loss of red cones in zebrafish (Brockerhoff *et al.*, 1997). These mutant fish, severely red-blind, have the other three cone types and respond normally to colors other than red. The gene defect is not, as in most human protanopes, in the red opsin gene, but in a gene that codes for a novel protein that may be involved in protein sorting and/or trafficking (Taylor *et al.,* 2005). Human disorders involving a single cone type have been identified only very rarely and are not at all well characterized. This mutation provides an animal model for such disorders as well as the possibility for elucidating further the mechanisms underlying protein sorting and trafficking in cones, about which we know little.

A third behavioral mutant has been shown to have a developmental defect affecting the development of the cone photoreceptor terminals (Allwardt *et al.*, 2001). These animals are completely blind behaviorally, but do demonstrate an ERG that is, however, highly abnormal. Processes from second-order cells do not penetrate into the cone terminals and thus the classic invaginated photoreceptor ribbon synapses fail to form in these animals. The mutation is in the gene that codes for synaptojanin, a protein that is involved in endocytosis at conventional synapses (Van Epps *et al.*, 2004). In developing photoreceptor terminals, this protein appears essential for forming the invaginated ribbon synapses, a process about which we know virtually nothing.

A developmental mutant called *young* has shown clearly that specification of the cells in the developing retina is distinct from the subsequent morphological differentiation of the cells (Fadool *et al.*, 1997; Link *et al.,* 2000). That is, in the mutant, cells are specified into various types (as shown by staining with cell-specific markers) but the nascent neurons fail to grow processes and make synapses. The mutation is in a gene that codes for a subunit of a chromatin remodeling complex, and thus is relatively uninformative with regard to the specific genes involved in the morphological

differentiation of retinal neurons during development (Gregg *et al.*, 2003). Nevertheless, this mutant has clearly demonstrated the specification and morphological differentiation are two distinct processes during development controlled by separate genetic control.

A dominant mutation that results in altered vision in adult fish and discovered by behavioral testing of adult fish has a defect in the centrifugal fiber input to the retina (Li & Dowling, 2000). In fish, this centrifugal fiber input is from the olfactory bulb and has been under study for many years by many laboratories. Whereas the synaptic contacts of these centrifugal fibers have been identified (mainly on the dopaminergic interplexiform cells) (Zucker & Dowling, 1987) and the substances released by the terminals identified (LHRH and FMRFamide) (Stell *et al.*, 1984; Umino & Dowling, 1991) the function of this centrifugal input has not been understood. Li and his colleagues have now shown that activation of the olfactory system by amino acids increases visual sensitivity as measured behaviorally providing insight on the role of these centrifugal fibers (Li & Maaswinkel, 2003). An interesting twist in the story is that the increase in visual sensitivity occurs only in the early morning hours when zebrafish are less sensitive to light because of a circadian rhythm depression of light sensitivity (Li & Dowling, 1998).

Many mutations affecting retinotectal development have also been described (Karlstrom *et al.*, 1996; Baier *et al.*, 1996), including one in which the ganglion cell axons project to the ipsilateral tectum rather than the contralateral tectum which is the norm. This mutant has a most curious behavioral defect that as yet is not well understood, but promises to tell us much about how eye tracking occurs. It reverses tracks moving stimuli; when stripes in its visual field move from left to right, its eyes move from right to left, and vice versa. Other retinotectal mutants show altered projection patterns as well as altered ganglion cell axonal terminal spreads.

In addition to contributing to retinal research, zebrafish are becoming increasingly useful in lens (Link *et al.*, 2001)and even glaucoma research. A number of mutations that affect lens development have been found and a mutant (called *bug eye*) that may be a model for glaucoma has been isolated (John *et al.*, 2003). Methods for measuring intraocular pressure in zebrafish have been developed (Link *et al.*, 2004; McMahon *et al.*, 2004), and it has been shown that the *bug eye* mutants have increased intraocular pressure.

The above examples represent only a few of the interesting mutants that have been uncovered so far. In many cases, the defective gene has been identified and these genes code for proteins that are involved in a variety of functions from chromatin remodeling to specific phototransduction roles (i.e. the G-protein transducin). The advantages of zebrafish for study of the eye, retina and visual pathways area many, and the results obtained so far suggest that this model system will become increasingly prominent in eye and vision research.

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National Cancer Institute (NCI)
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The zebrafish is a well-established system for the analysis of vertebrate embryogenesis, organogenesis and disease, and zebrafish have been shown to share conserved pathways of development and disease pathogenesis with humans. Its powerful forward-genetic capacity permits the identification of novel genes that are activated (oncogenes) or inactivated (tumor suppressors) during malignant transformation. Transgenic strategies can be used to generate conditional zebrafish models of leukemias and solid tumors and these can be used as the basis for forward genetic modifier screens.

Through mutagenesis screens, genes regulating myelopoiesis are being studied for contribution to the molecular pathogenesis of myelodysplastic syndrome and acute myeloid leukemia. Similar approaches are being used to study peripheral sympathetic nervous system (PSNS) development and to identify tumor suppressors in childhood neuroblastoma. A tumor-prone, mutant p53 zebrafish line, has been obtained in order to study pathways involving his key regulator of DNA repair, cell cycle regulation and apoptosis. p53 is the most commonly mutated gene in human cancers, and the zebrafish model is allowing the study of its role in several types of cancer.

A transgenic line that develops T-cell acute lymphoblastic leukemia, (T-ALL) has been created by expressing c-myc, and a melanoma model has been made from a combination of BRAF activation and p53 deficiency. These fish are being used to conduct one of the first "cancer-related" vertebrate genetic modifier screens, which will allow us to identify enhancers and suppressors of T-ALL progression. Through the combined use of these approaches, we hope to uncover novel genes and targets for the development of small molecule inhibitors and eventually improved therapeutic drugs.

The pathways that initiate programmed cell death or apoptosis are being studied. By use of a BCL2-GFP transgenic zebrafish line, a forward genetic screen for modifiers of the BCL2 pathway is being conducted. This will test the ability of these mutations to accelerate or delay the onset of leukemia in our leukemia model. Mutations identified in this screen may identify novel targets for the development of small molecule inhibitors to treat follicular lymphoma and other human cancers.

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Investigation of animal models such as the zebrafish provides key insight toward improving oral, dental and craniofacial health. Dental disease affects essentially our entire population. Cleft palate heads the list of common birth defects. About 18% of all Americans (and an alarming 70% of women over age 65) will suffer from osteoarthritis during the current decade, a disorder that prominently includes misregulation of the cartilage-endochondral bone developmental pathway. Work with zebrafish in particular during the past decade has established that many of the genes and their functions that control such pathways have been highly conserved not just in mammals, but in all vertebrates. Attributes of the zebrafish for mutational analyses facilitate identifying these genes by their functions. Zebrafish have remarkable advantages for study of craniofacial development as well.

The cranial neural crest is a key lineage that forms craniofacial bone and teeth, in zebrafish and humans alike. In zebrafish this lineage includes relatively very few cells and develops rapidly, forming a primordial head skeleton within about 4 days after fertilization, that consists of well-developed cartilage, bone, and teeth. Because fertilization is external, and the embryos can be easily cultured, and because they are optically transparent, the whole process of head skeletal development can be recorded in real time *in vivo*, with single cell resolution. A single cell can be marked with dye and its developing lineage watched. Single cells can be transplanted between embryos, providing a crucial way to learn in what specific cells a gene must function correctly for skeletal development to occur correctly. These features, including genetic conservation and the attributes of the zebrafish for genetic study and developmental observation and manipulation, combine to make the zebrafish one of the most outstanding models for understanding the fundamentals of most aspects of human craniofacial development.

Ongoing projects with zebrafish include investigation of the initial specification and formation of the cranial neural crest, the interactions of the crest-derived skeleton-forming cells with their substrates during morphogenesis, and the examination of the histogenesis of dermal bone. Critical in the regulation of each of these processes is intracellular signaling between skeleton-forming cells, or between these cells and other cells in their environments. The molecules mediating the signaling are being studied as well in zebrafish, including fibroblast growth factors in tooth development, and bone morphogenetic proteins and endothelins in cranial cartilage and bone development. Some of these molecules, including endothelin1, appear to function as morphogens, where the level of signaling specifies what type of skeletal element will develop Hundreds of craniofacial mutations are available, and more continue to be produced in ongoing screens, their study providing significant insight into disease states such as holoprosencephaly. Some mutants have quite delicate skeletal disturbance phenotypes, allowing extremely precise understanding of the underlying cellular interactions. Excellent progress in the mutational analyses of craniofacial development will continue to depend on advances in zebrafish genomics.

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